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RESEARCH ARTICLE

Dispersal constraints for the conservation of the grassland herb *Thymus pulegioides* L. in a highly fragmented agricultural landscape

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Abstract Species-rich grassland communities are one of the most important habitats for biodiversity and of high conservation priority in Europe. Restoration actions are mainly focused on the improvement of abiotic conditions, such as nutrient depletion techniques, and are generally based on the assumption that the target community will re-establish at the restored site when the target species exist in the neighborhood. Information on the contemporary seed-dispersal range is therefore crucial to develop effective conservation measures. Here, we investigated the contemporary long-distance seed dispersal and genetic structure of the grassland herb *Thymus pulegioides* in an intensively managed agricultural landscape in Flanders (Northern Belgium). Assignment tests based on amplified fragment length polymorphisms revealed very low levels of effective seed dispersal between populations although seed availability and seed viability was not a limiting factor. The process of fragmentation has resulted in a high population differentiation and without further incoming gene flow the remnant populations are prone to further genetic erosion and perhaps extinction. Our findings illustrate that restoring suitable abiotic habitat conditions in the neighborhood of existing populations does likely not guarantee colonization for this grassland specialist. For the survival of the species, existing populations should be functionally connected and seed addition may be necessary for successful conservation to overcome dispersal-limitation.

Keywords Genetic diversity · Habitat fragmentation · Functional connectivity · Seed dispersal · *Thymus pulegioides* L.

Introduction

Species-rich, semi-natural grassland communities are one of the most important habitats for biodiversity and of high conservation priority in Europe. These grasslands are the

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remnants of habitats created by low-intensity, traditional farming, or, in some cases, the natural vegetation on poor soils or in exposed locations (Pigott and Walters 1954). Large scale abandonment of traditional agricultural practices followed by agricultural intensification including intensive cutting or grazing, re-sowing of plants and inorganic fertilizer use, has driven an unprecedented loss of species-rich grasslands across Europe in the past decades (Veen et al. 2009; Walker et al. 2004). Consequently, semi-natural grasslands have become increasingly restricted to small and isolated patches (Veen et al. 2009). Owing to decreased population size in these small habitat patches, populations of characteristic grassland plant species are frequently subject to high genetic drift (e.g. Hooftman et al. 2004; Jacquemyn et al. 2010). Furthermore, increased spatial isolation may limit seed and pollen dispersal to below a critical threshold which decreases the potential to counteract genetic drift and inbreeding through gene flow (e.g. Bijlsma and Loeschcke 2012; Honnay and Jacquemyn 2007). The connectivity of semi-natural grasslands has been severely reduced by the cessation of traditional grazing and mowing practices of these grasslands which formerly supported seed dispersal between plant populations through movement of livestock and man (e.g. harvesting and sowing) (Bakker and Berendse 1999; Poschlod et al. 1998; Rico et al. 2014). High levels of genetic erosion may result in increased extinction risk through inbreeding depression (Aguilar et al. 2008; Becker et al. 2011), loss of adaptability to changing environmental conditions (Willi et al. 2006) or increased susceptibility to pathogens (Luquet et al. 2012). Consequently, it can be expected that conservational efforts of small species-rich, semi-natural grasslands fragments are constrained by the genetic diversity of the constituent characteristic plant populations (Oostermeijer et al. 2003).

Policies have been introduced that encourage the conservation and restoration of these species-rich grasslands (Bakker and Berendse 1999; Helsen et al. 2013; Jacquemyn et al. 2010). Typical conservation practices as potential ways for reducing fragmentation effects are the passive protection of remaining sites, restoring habitat patches and the establishment of corridors and stepping stones to enhance migration and dispersal for specific target species. It is generally assumed that these conservation practices will likely be effective if the target community already exist in the neighborhood so that seeds can be dispersed by the local species pool. Whether these practices are effective in restoration largely depend on the fecundity, abundance and dispersal capacity of the target species (Bakker and van Dam 1999; Rico et al. 2012; Whitlock and McCauley 1999). However, most of the research in landscape ecology has focused more upon elements of spatial explicitness than on the biology of living organisms (Murphy and Lovett-Doust 2004). Information on the contemporary dispersal range and the functional connectivity of populations is crucial to the development of effective conservation measures (Baguette et al. 2013; Bullock et al. 2006). For grassland specialist plants, it is generally assumed that they are dispersal-limited (Bakker and van Dam 1999; Eriksson 1998) but there are few studies that quantify this process (but see Jacquemyn et al. 2010). Most former population genetic studies estimated migration rates based on indirect measures such as Wright's F_{ST} (Storfer et al. 2010; Whitlock and McCauley 1999). F_{ST} is an excellent measure for the genetic differentiation between populations but should not be used for estimating current long-distance dispersal events because it also reflect the genetic signature of historic gene flow (Baguette et al. 2013; Whitlock and McCauley 1999). Individual-based methods such as spatial assignment tests, as opposed to clustering methods, can be used to identify recent inter-population gene exchange during the last generations. Despite their potential, assignment tests have rarely been used for this purpose (Aavik et al. 2013), largely due to the inherent difficulty to identify and sample all the fragments in a given landscape (Kamm et al. 2009). Amplified fragment length polymorphisms (AFLPs) are efficient markers for assigning each individual to its population (e.g. Campbell et al. 2003; He et al. 2004; Vanden Broeck et al. 2014) and, given a comparable analytical effort in the lab, much more efficient than microsatellite markers in discriminating the source of an individual among putative source populations, especially at intermediate spatial scales (Campbell et al. 2003). Here we investigate the contemporary, effective seed dispersal that occurred during the last living generations of the grassland herb *Thymus pulegioides* (broad-leaved thyme) in an intensively managed agricultural landscape in Flanders (Northern Belgium). Broad-leaved thyme is 'specialized' to semi-natural grasslands in the study area, i.e. it occurs in similar vegetation along road verges and in remnant populations in areas that were previously semi-natural grasslands, while not generally occurring in the dominant landscape matrix (Cousins and Eriksson 2001). This makes broad-leaved thyme an interesting species for studying the ability of grassland specialist plant species to persist in the present-day landscape. Populations of broad-leaved thyme dramatically decreased in Flanders during the last decades (Van Landuyt et al. 2006). The probability of colonization of the restored habitat will largely depend on the fecundity and the seed dispersal potential of the target species. Former studies indicate that *Thymus* species are likely dispersal-limited with most of the seeds falling close to the parent plant (Belhassen et al. 1987; Eriksson 1998; Pigott 1955; Tarayre et al. 1997). However, these studies have focused on short distance dispersal (Belhassen et al. 1987; Pigott 1955), on regional distribution patterns

(Eriksson 1998) and on indirect estimates of gene flow based on the genetic differentiation between populations (Wright's F_{ST}) (Tarayre et al. 1997), but they were not designed to detect the rare long-distance seed dispersal events that may contribute to colonization and functional connectivity among populations. We used AFLPs to estimate recent patterns of seed dispersal among populations and to investigate the genetic diversity and structure. In addition we investigated seed availability and viability in a greenhouse experiment. We hypothesized that poor seed dispersal is a main limiting factor in functional population connectivity.

Materials and methods

Study species and sampling sites

Thymus pulegioides is a small, diploid (2n = 28), perennial forb (family Lamiaceae) that grows in open, unshaded habitats on well-drained, low-nutrient soils throughout Europe, Asia and North Africa (Javadi et al. 2009; Pigott 1955). The species is quite specialized in its habitat requirements, depends on habitat disturbance for its recruitment and is very susceptible to increased competition for light (Ouborg et al. 2006). Female and hermaphrodite individuals occur together in natural populations (i.e. gynodioecy) with the hermaphrodites being self-compatible (Pigott 1955). Seedlings frequently grow on small hillocks built by either moles or ants (Bonte et al. 2003). Mature plants of broad-leaved thyme flower each year from July to August and the plants are pollinated by insects, mainly solitary bees (Pigott 1955). The seeds ripen by September but the dead flowering stems remain standing throughout the winter and may still contain viable seeds in the following spring (Pigott 1955). Fresh, fully swollen seeds have a high germination percentage (80–100 %) (Pigott 1955). Studies quantifying seed dispersal distances are lacking but observations suggest that seeds are generally dispersed within a meter from the parent plant (e.g. Pigott 1955; Walker et al. 2004). Wind dispersal of whole inflorescences may also occur (Pigott 1955). Secondary, horizontal seed dispersal may occur by ants (myrmecochory) (Becker et al. 2011; Bonte et al. 2003) or through animal intake (endozoochory) or external animal dispersal (epizoochory) (Cosyns et al. 2005). Based on former studies, Thompson et al. (1997) classify the seed bank of broad-leaved thyme twice as transient, twice as short-term persistent, and three times as long-term persistent. Vegetative reproduction by runners or stolons is common. Under heavy grazing, the runners may be disrupted resulting in a group of separate individuals representing the same genotype (Pigott 1955). In the absence of grazing, mature plants form a small tangled cushion (Pigott 1955). Roots have been found to persist up to 13 years (Pigott 1955).

Twenty locations representing all known populations of broad-leaved thyme in central-east Flanders (northern Belgium), were included in this study (Fig. 1). The study area is characterized by sandy to loamy, moderately to well buffered soils. The area was historically covered by large stretches of heathland and species-rich grasslands that were traditionally managed through extensive grazing, burning and hay cutting. From the nineteenth century onwards, changes in traditional farming practices towards more intensive agriculture have led to losses of many of these species-rich grasslands (Van Landuyt et al. 2006). Nowadays, the species is only represented by isolated, often extremely small relict populations, mainly growing along roadsides on sunny talus with a southern exposure. More information on the sampling locations is given in Table 1 and in Fig. 1.

Fecundity characteristics and population size

Fecundity characteristics like seed availability and viability, determines the dispersal ability. Seed weight, seed germination percentage and seed germination speed were used as measures of fecundity characteristics. Increased seed weight and the early emergence of seeds increases fitness components of seedlings such as survival and growth (Verdu and Traveset 2005). In September and October 2012, seeds were collected within each sampled location, unless inflorescences were absent as a result of recent mowing or grazing (Supplementary Table 1). We collected 60 seeds randomly selected from flowering individuals within each site. Seed weight was investigated by weighting 20 randomly selected seeds per site. Seeds were placed in Petri dishes on Whatman No. 1 filter paper with water, put in a greenhouse at a constant temperature at 21 °C and seed germination percentages were recorded daily until 3 days after it was observed that seeds stopped germination (this was after 27 days). The census population size was estimated by recording the total area covered by broad-leaved thyme cushions for each location.

DNA extraction and AFLP analysis

In August 2012, we sampled all individual plants forming spatially separated cushions within each of all the 20 known locations, except for two large populations where sampling was restricted to a maximum of 54 samples (population code ZEL and TON: Table 1). This resulted in an average of 21 sampled individuals per location but this number ranged from 2 to 54, due to small census population sizes. In total, we sampled 417 individuals. Five to seven young leaves were collected from each sampled

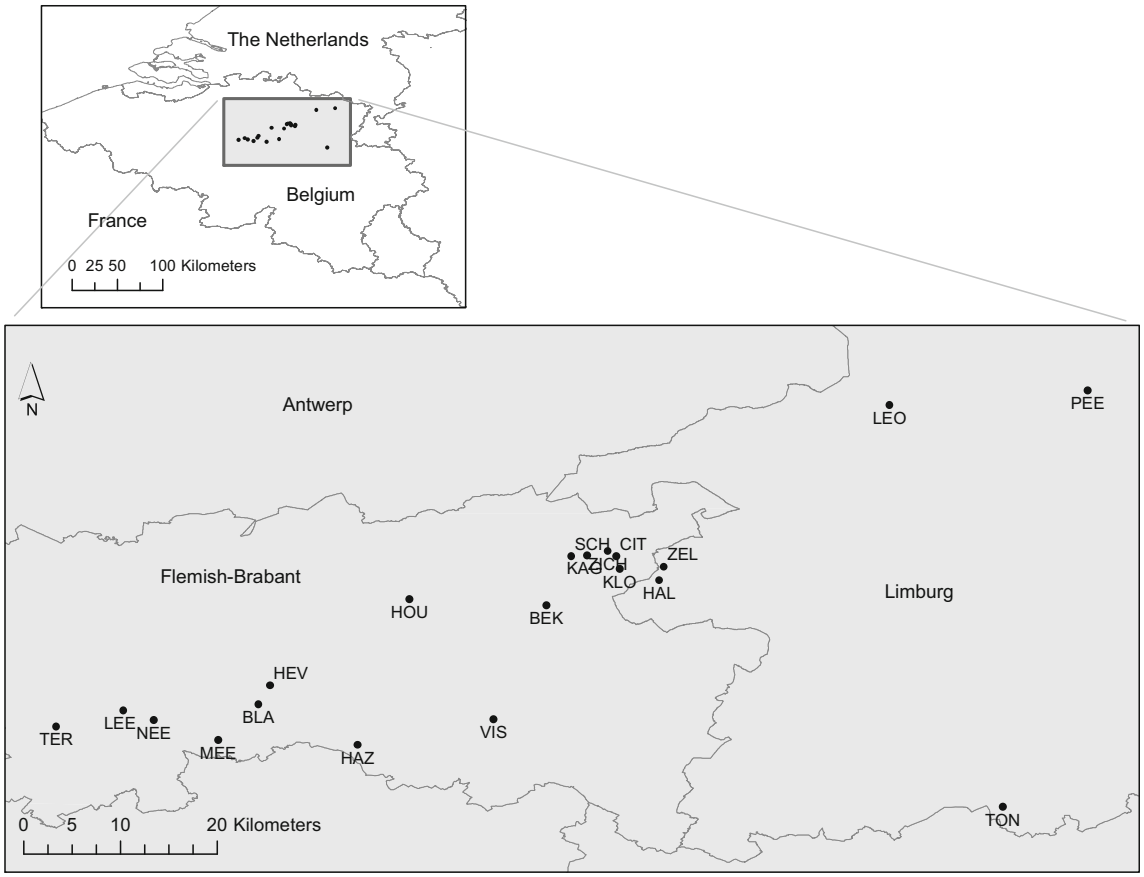


Fig. 1 Map of the study area and of *Thymus pulegioides* sampling locations

plant. Total DNA was extracted from silica dried leaf samples with the QuickPickTM Plant DNA kit (Isogen Life Science, De Meern, The Netherlands). AFLP-fingerprints were generated according to Vos et al. (1995), with restriction and ligation conducted in one single step. After preliminary tests with 17 primer pairs, two primer combinations (*Eco*RI-ACT/*Mse*I-CAC, *Eco*RI-ACC/*Mse*I-CTA) were chosen which resulted in clear bands of sufficient variability. PCR products from each primer pair were run on an ABI 3500 capillary sequencer (Applied Biosystems). Genescan 600-Liz (PE Applied Biosystems) was used as an internal lane size standard. Raw data were sized with GeneMapper 4.1 (Applied Biosystems). To test for reproducibility, 25 (10 %) samples were randomly chosen and replicated from the DNA-extraction step. A binary matrix of AFLP band presence (1)—absence (0) was built using the automated scoring package RawGeno v 2.0 (R CRAN (Arrigo et al. 2009)) using the scoring parameters: MINBIN = 1, MAXBIN = 2, FREQ = 1, THRESH = 80. The replicated samples allowed the removing of non-reproducible bins and subsequently, the calculating of the error rate with RawGeno v 2.0 according to the method of Bonin et al. (2004). As recommended by Vekemans et al. (2002) the correlation between AFLP band size and frequency among samples was assessed for each primer combination to check for potential homoplasy. Before performing further analysis, we excluded loci with frequencies below 5 % and above 95 % that may lead to spurious correlations and are therefore not considered reliable (Roesti et al. 2012). Linkage disequilibrium among AFLP loci was tested using pairwise logistic regressions. We used the false discovery rate (FDR) based multiple comparison procedure (Benjamini and Hochberg 2000) to correct for multiple testing. The maximum FDR was set to 5 %. The calculations were performed using the R packages fdrtool 1.2.10 (Strimmer 2008) and brainwaver 1.5 (http://cran.r-project.org/web/packages/brainwaver/).

Data analysis

As *Thymus* can reproduce vegetatively, we first verified whether some genotypes occurred more than once within a population. Small genotyping differences between ramets representing the same genet could appear as a result of mutations and scoring errors. The number of distinct genets occurring in the sample (G) was inferred by examination

Table 1 Description of the 20 sampling populations of *Thymus pulegioides*

Code	Population	Area (m^2)	N	N_{AFLP}	G	Pd	PPL	H_j	DW	F_{IS}
BEK	Bekkevoort	28.03	39	38	38	1.00	75.5	0.28	0.45	0.05
BLA	Blanden	0.82	29	23	10	0.43	79.4	0.283	0.37	0.27
CIT	Citadel Diest	5.53	35	31	29	0.94	71.6	0.257	0.41	0.10
HAL	Halen	0.03	5	5	1	0.20	NA	NA	0.45	0.23
HAZ	Hazenberg	1.70	7	5	2	0.40	47.7	0.198	0.44	0.15
HEV	Heverlee	0.69	10	8	8	1.00	69	0.256	0.51	0.24
HOU	Houwaart berg	0.62	12	11	8	0.73	62.6	0.229	0.42	0.17
KAG	Kaggevinne	0.37	7	5	3	0.60	45.8	0.194	0.35	0.32
KLO	Kloosterberg	0.26	5	4	1	0.25	NA	NA	0.42	0.20
LEE	Leefdaal	2.75	10	6	5	0.83	63.2	0.252	0.56	0.10
LEO	Leopoldsburg	3.08	32	26	21	0.81	83.9	0.293	0.48	0.29
MEE	Meerdalwoud	0.54	42	37	32	0.86	61.3	0.213	0.36	0.35
NEE	Neerijse	0.93	21	18	8	0.44	81.9	0.307	0.46	0.52
PEE	Peer	0.12	3	2	2	1.00	55.5	0.243	0.61	0.05
SCH	Scherpenheuvel	2.38	16	14	2	0.14	42.6	0.175	0.3	0.12
TER	Tervuren	0.32	6	5	3	0.60	62.6	0.287	0.52	0.44
TON	Tongeren	21.10	54	52	47	0.90	81.3	0.288	0.43	0.18
VIS	Vissenaken	14.47	32	29	29	1.00	74.2	0.263	0.45	0.01
ZEL	Zelem	1.07	50	44	38	0.86	67.7	0.24	0.44	0.15
ZIC	Diest	0.15	2	0	NA	NA	NA	NA	NA	NA
Mean		4.248	20.8	18.15	15.1	0.83	66.22	0.25	0.44	0.20
Total		84.96	417	363	287	0.79				

Area estimated total area occupied by *Thymus pulegioides* cushions, *N* number of sampled individuals, N_{AFLP} number of samples fully genotyped by AFLPs, *G* the number of genets, *Pd* genotypic richness (=G/N_{AFLP}), *PPL* percentage of polymorphic loci at the 5 % level, H_j expected heterozygosity, *DW* frequency-down-weighted marker values, F_{IS} mean inbreeding coefficient, *NA* not available

the histogram of the frequency distribution of pairwise genetic distances based on the simple matching coefficient using AFLPDAT (Ehrich 2006). As identical genotypes can also be produced under sexual reproduction when the amount of genetic variation is extremely low, we tested the probability of finding the observed clonal diversity under random mating with GenoDive 2.0b24 (Meirmans and Van Tienderen 2004), using the corrected Nei's diversity index as test statistic and with a randomization of alleles over individuals within populations based on 999 permutations. Duplicate genets were removed prior to further analysis to ensure independence of the samples.

We estimated individual inbreeding level using AFLP-calc (Dasmahapatra et al. 2008), a method developed for unlinked, biallelic dominant markers assuming that at least half the individuals are outbred. Allele frequencies were estimated with AFLP-SURV v 1.0 (Vekemans et al. 2002) using a Bayesian approach and a non-uniform prior distribution following Zhivotovsky (1999), using the estimate for the mean inbreeding coefficient (F_{IS}) as calculated with AFLPcalc. Genetic diversity was investigated by quantifying the: genotypic richness (Pd), the proportion of polymorphic loci (PPL) at the 5 % level and Nei's gene diversity (H_j, which is analogous to H_e) (Lynch and Milligan 1994). Frequency down-weighted marker values (rarity index or DW-values) (Schonswetter and Tribsch 2005) were calculated with AFLPDAT (Ehrich 2006).

As a measure of population differentiation we calculated F_{ST} using AFLP-SURV v 1.0 using 100 permutations. In addition, pairwise population Φ_{PT}-values were estimated using AMOVA in GenAlEx 6.4 (Peakall and Smouse 2006). Significance was calculated using the available Monte Carlo procedure (999 permutations). Genetic structure was further investigated by a principal coordinates analysis (PCoA), also performed with GenAlEx 6.4. To check for a significant pattern of isolation-by-distance (IBD), a Mantel test between pairwise Φ_{PT}-values and pairwise geographic distances was performed as implemented in GenAlEx 6.4 (99 permutations) for the populations that contained more than 20 distinct genets (Table 1). In addition, we investigated the presence of spatial patterns in genetic variation with principal coordinates of neighbor matrices (PCNM) on the detrended genetic data (Borcard and Legendre 2002).

To estimate contemporary gene flow by seed between populations, we used population likelihood assignment

tests for individuals. We therefore applied the procedure of Duchesne and Bernatchez (2002), developed for AFLP markers and implemented in AFLPOP v.1.1. This approach is based solely upon AFLP band frequencies and the assumptions that frequency estimates per population are accurate and that the loci are statistically independent. AFLPOP identifies for a given genotype and a set of sampled populations, the most likely source population. A minimum log-likelihood difference (MLD) of 1 was used to assign specimens to the most likely population (re-allocation procedure). This means that a genotype has to be ten times more likely to be found in a given population than in any other population in order to be assigned to that population. In case MLD's are smaller than one, individuals could not be assigned unambiguously to one of the sampled populations. Because log(0) is not defined, frequencies of zero need to be replaced by an appropriate value. As recommended by Campbell et al. (2003), we chose 1/(n + 2) as the substitution value with n the sample size. Because small sample sizes can result in large errors in the estimation of allele frequencies (Campbell et al. 2003), we excluded the populations with less than 5 genotypes (8 populations) resulting in the re-allocation of 268 individuals from 11 populations. We assessed the probability of incorrect assignment using the AFLPOP simulator with 10 iterations and MLD = 1. This procedure generates 1,000 random progeny at each iteration, based on the samples, and outputs the proportion of occurrences (estimate of *P*) of allocation to the second population. When the simulated *P* is low, an incorrect allocation by chance alone to a population other than that from which they were sampled is extremely unlikely. When *P*-values for a source-unknown individual are < 0.001 for all candidate populations, it is very likely that the individual comes from populations other than those that were sampled (He et al. 2004).

Finally, we performed simple linear regressions to identify possible relationships between genetic diversity (measured in terms of PPL, F_{IS}, H_j and log-transformed G) as response variables, and fitness characteristics (log-transformed population size, seed germination percentage and seed germination speed) as exploratory variables.

Results

Fitness characteristics and population size

The mean census population size estimated by the area covered by broad-leaved thyme cushions was 4.5 m^2 (range: 0.030–28.03). Seeds could be collected from 16 out of the 20 populations that contained flowering plants. Mean germination percentage per population ranged from 0 to 68.33 % (overall mean: 18.95 %), mean seed weight per 100 seeds ranged from 0.0030 to 0.026 g (overall mean: 0.011 g) and mean seed germination speed ranged from 0 to 0.81 germinated seeds per day (overall mean: 0.23 seeds per day) (Supplementary Table).

Genetic diversity and long-distance seed dispersal

The two primer combinations resulted in a clear AFLP-profile of 155 polymorphic markers for 363 out of the 417 samples (87 %) with a mean genotyping error following Bonin et al. (2004) of 4.7 % per locus. The 54 samples with incomplete AFLP-profiles, including the two individuals of the location ZIC, were discarded from further analysis. This resulted in genotyped individuals with complete AFLP-profiles for 19 out of the 20 known locations of broad-leaved thyme in central-east Flanders (northern Belgium) (Table 1). No significant correlation between fragment sizes and frequencies was found (*Eco*RI-ACT/*Mse*I-CAC; $r^2 = -0.223$, $P = 0.07$. *Eco*RI-ACC/*Mse*I-CTA; $r^2 = -0.127$, $P = 0.12$) indicating that the potential bias on estimates of genetic diversity due to size homoplasy was low. Pairwise logistic regressions between the 155 loci were significant for 6.9 % of all comparisons ($P < 0.0005$), suggesting that less than 7 % of all pairwise loci comparisons were not independent. The frequency distributions of pairwise genetic distances between samples collected within a population showed a bimodal genetic distance distribution with a 'left peak' near zero (Fig. 2), which indicates the presence of duplicate genotypes. The results of the test for clonal population structure indicate

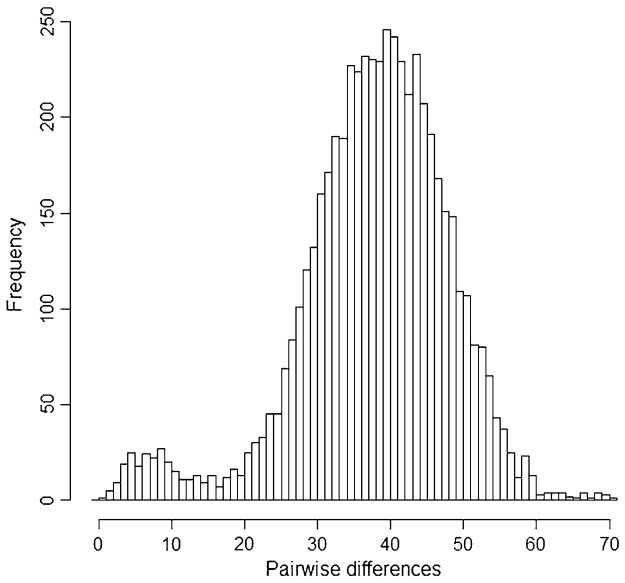


Fig. 2 Frequency distribution of pairwise genetic distances between individuals of *Thymus pulegioides* within the sampling sites and calculated from the simple matching coefficient

that the observed genotypic structure cannot be explained by sexual reproduction ($P = 0.001$), and is therefore likely caused by clonal reproduction. Based on this histogram, genotypes differing at more than 12 loci were considered to belong to different clones or genets (Fig. 2). Genotypes differing at less than 12 loci were considered as identical clones (ramets of the same genet) and all but one was removed for further data analysis. This resulted in 287 unique genets. The number of genets per population is given in Table 1.

The estimated mean inbreeding coefficient (F_{IS}) for the 287 genets was 0.20. This corresponds with a mean selfing rate (s) of 0.33. The PPL ranged from 20.0 to 74.30 with a mean of 53.2, H_j ranged from 0.10 to 0.24 with a mean of 0.19 (Table 1). Two singletons (populations with only one genet: HAL and KLO) were removed for the analyses of population differentiation resulting in 285 genets from 17 populations. F_{ST} (mean ± SD) was 0.23 (± 0.097) whereas the mean pairwise Φ_{PT} was 0.26 and significantly greater than zero ($P = 0.01$; permutation test with 999 repetitions). Pairwise Population Φ_{PT}-values for the populations that contained more than 20 distinct genets are represented in Table 2. We detected no significant IBD-pattern ($r = -0.16$, $P = 0.50$) (Fig. 3). No significant spatial structure was detected by the PCNM analysis ($P = 0.64$). PCoA revealed that most populations grouped together in one cluster, except for the populations MEE, ZEL, BEK and VIS that cluster more or less apart from the rest indicating distinct gene pools (Fig. 4).

Table 2 Pairwise population Φ_{PT}-values for the populations that contained more than 20 distinct genets

	Bek	Cit	Leo	Mee	Ton	Vis	Zel
Bek	0.000	0.001	0.001	0.001	0.001	0.001	0.001
Cit	0.173	0.000	0.001	0.001	0.001	0.001	0.001
Leo	0.209	0.219	0.000	0.001	0.001	0.001	0.001
Mee	0.339	0.332	0.290	0.000	0.001	0.001	0.001
Ton	0.168	0.151	0.182	0.258	0.000	0.001	0.001
Vis	0.221	0.229	0.244	0.352	0.160	0.000	0.001
Zel	0.257	0.227	0.278	0.315	0.211	0.288	0.000

Φ_{PT}-values below the diagonal. Probability, P(rand ≥ data) based on 999 permutations is shown above the diagonal

Assignment tests allocated 261 (97.3 %) individuals to a single genetic population. Of these, 258 (98.9 %) were assigned to the population from which they were sampled and three (1.1 %) individuals were identified as genetic outliers and were allocated to another population than the sampling population. They included the following individuals: one sampled in BEK and assigned to CIT (distance between populations: 6.8 km), one sampled in LEO and assigned to BLA (51.4 km) and one sampled in CIT and assigned to VIS (18.6 km). Increasing the stringency of the assignment decision criterion to MLD = 2 (i.e. a genotype has to be 100 times more likely to be assigned to a given population) reduced the number of putative migrants from three to two; the sample from LEO that was assigned to BLA under MLD = 1 could not be allocated confidently under MLD = 2. The simulation analysis resulted in high statistical support for the individual assignments. The probability that an individual was allocated to other populations by chance alone under MLD = 1 was very low ($P = 0.001$ or 99.9 % success rate). No confident assignment was possible for 7 individuals (2.7 %).

Significant positive correlations were detected between the total area covered by the population and the estimated genetic diversity parameters Hj, PPL and log (G). Negative significant correlations were detected between the mean

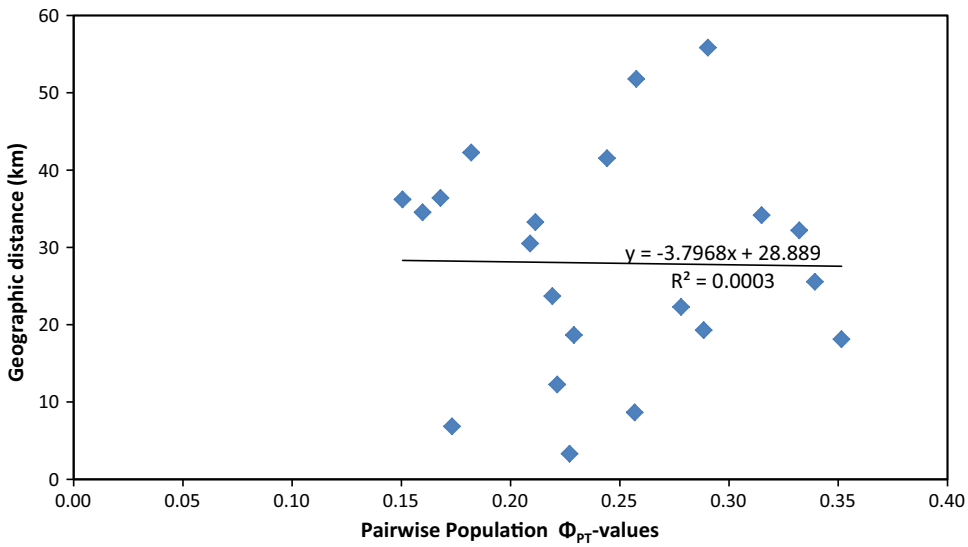


Fig. 3 Pairwise Population Φ_{PT}-values plotted and regressed against geographic distances for the seven populations of *Thymus pulegioides* with more than 20 individuals analyzed

seed germination speed and the estimated mean genetic diversity parameters F_{IS} and log (G), and between the mean seed germination percentage and F_{IS} (p-values < 0.05, Table 3).

Discussion

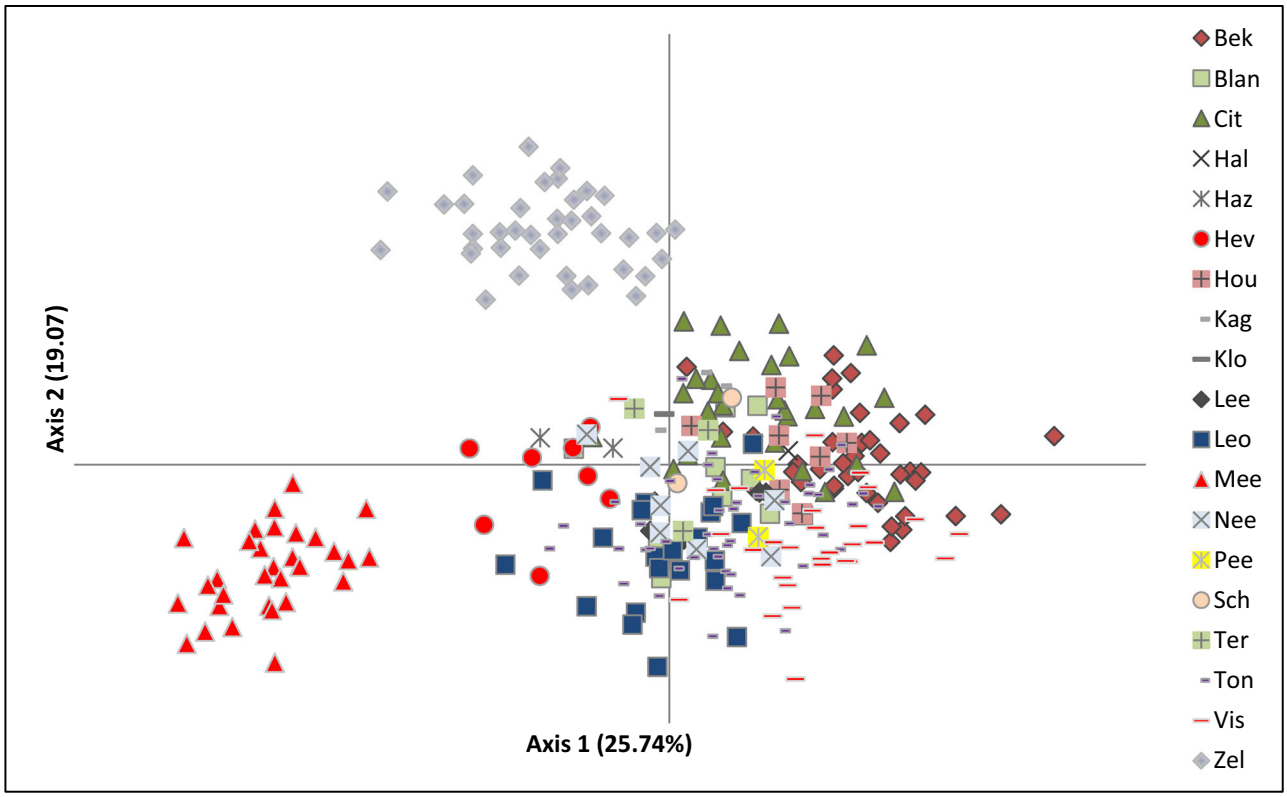
We hypothesized that poor contemporary population connectivity and small population size in the highly fragmented landscape, threatens the long term conservation of isolated populations of *T. pulegioides*. Indeed, we found extremely low inter-population gene flow as only a few individuals (0.7–1.1 %) appeared to have originated from other populations and were classified as putative migrants. We further interpret these putative migrants as a consequence of seed dispersal events, as it is unlikely that effective pollen flow could generate such a high genetic resemblance (ten times more likely) with another population (Albaladejo et al. 2009; He et al. 2004). These few putative migrants might be the result of long-distance dispersal by wildlife (rabbits, roe-deer, foxes or rodents) or by mowing machinery. However, it is also possible that they originate from dispersal events that occurred up to several decades ago, when the species was much more common in the study area and when populations were larger and more connected. Some genotypes may have persisted several decades in the currently isolated populations owing to the ability of *T. pulegioides* to reproduce clonally.

Beside the low potential of long-distance seed dispersal, low functional population connectivity may also be caused by reduced fecundity. In this study, we collected seeds from each population with inflorescences (16 out of 20 populations) and observed a mean seed germination percentage of 19 %. This seed germination percentage is not particularly low as for *Thymus* sp. only rarely all four nutlets in a single calyx are viable (Pigott 1955). Hence, seed availability and seed viability were likely not strong limiting factors for long-distance dispersal. The extremely low or absent contemporary seed-dispersal observed is in concordance with former studies on *Thymus* species suggesting that seeds are generally dispersed within a meter from the parent plant (e.g. Pigott 1955; Tarayre et al. 1997). Insect-borne gene flow among the populations through pollen is also unlikely because pollen dispersal by bees is generally restricted to a few hundred meters (Pasquet et al. 2008). In addition, pollen movement over longer distances is unlikely as *T. pulegioides* in the study area only occurs in very small populations which attract less pollinators (Pasquet et al. 2008).

Most populations tend to cluster together in the plot of the PCoA but there are exceptions like the populations MEE, ZEL, BEK and VIS that cluster more or less apart from the rest, indicating distinct gene pools. The admixture between most of the populations of broad-leaved thyme observed in the PCoA likely reflects historical population connectivity when broad-leaved thyme was widespread in the study area. Before the nineteenth century when shepherding was common and occurred over large distances (>100 km), dispersal was likely not a limiting factor for characteristic grasslands species (Poschlod et al. 1998). Sheep especially are known as efficient dispersal vectors for most of the actual species in grasslands, dispersing seeds mainly through their hoofs and fur (e.g. Fischer et al. 1996; Rico et al. 2014). Human-induced habitat fragmentation, strong reductions in population size and local differences in the effect of genetic drift rather than long-term isolation of the present populations might have produced the distinctive gene pools observed in the PCoA. This may also explain the high population differentiation (mean $F_{ST} = 0.23$/mean $\Phi_{PT} = 0.26$, range of pairwise population Φ_{PT}-values for populations that contained more than 20 distinct genets: 0.15–0.32). However, for the population MEE the high pairwise population Φ_{PT}-values may also be the result of a founder event following an unintentional introduction of seed or root fragments during the recent establishment of a nearby gravel road using gravel and stone debris from a location in southern Belgium where broadleaved-thyme is more common. It is therefore possible that MEE is not an autochthonous population and thus not a relic of the historical metapopulation of the study area.

The findings of this study are in agreement with former studies indicating that grassland specialists are frequently dispersal-limited (Helsen et al. 2013; Pywell et al. 2002). However, some level of dispersal and colonization is essential for the persistence of populations and for the long-time survival of the species (Bolker and Pacala 1999; Nathan et al. 2003). Low functional connectivity between populations inevitably results in elevated inbreeding which in turn may reduce the viability of populations (Young et al. 1996). Accordingly, we found a relative high mean inbreeding coefficient ($F_{IS} = 0.20$) in the absence of a significant isolation-by-distance effect. Significant negative correlations (p value < 0.05) between the population inbreeding coefficient F_{IS} and the mean population seed viability suggest inbreeding depression resulting from a lack of regional equilibrium between gene flow and drift with drift being more influential than gene flow (Hutchison and Templeton 1999). It has to be mentioned that our results are inferred from dominant markers which have lower powers than co-dominant markers like

Fig. 4 Biplots of the principal component analysis of pairwise Euclidean genetic distances calculated for 287 individuals of *Thymus pulegioides* collected in 19 populations in Flanders and based on 155 polymorphic AFLP markers


Axis 2 (19.07)
Axis 1 (25.74%)
Bek
Blan
Cit
Hal
Haz
Hev
Hou
Kag
Klo
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Leo
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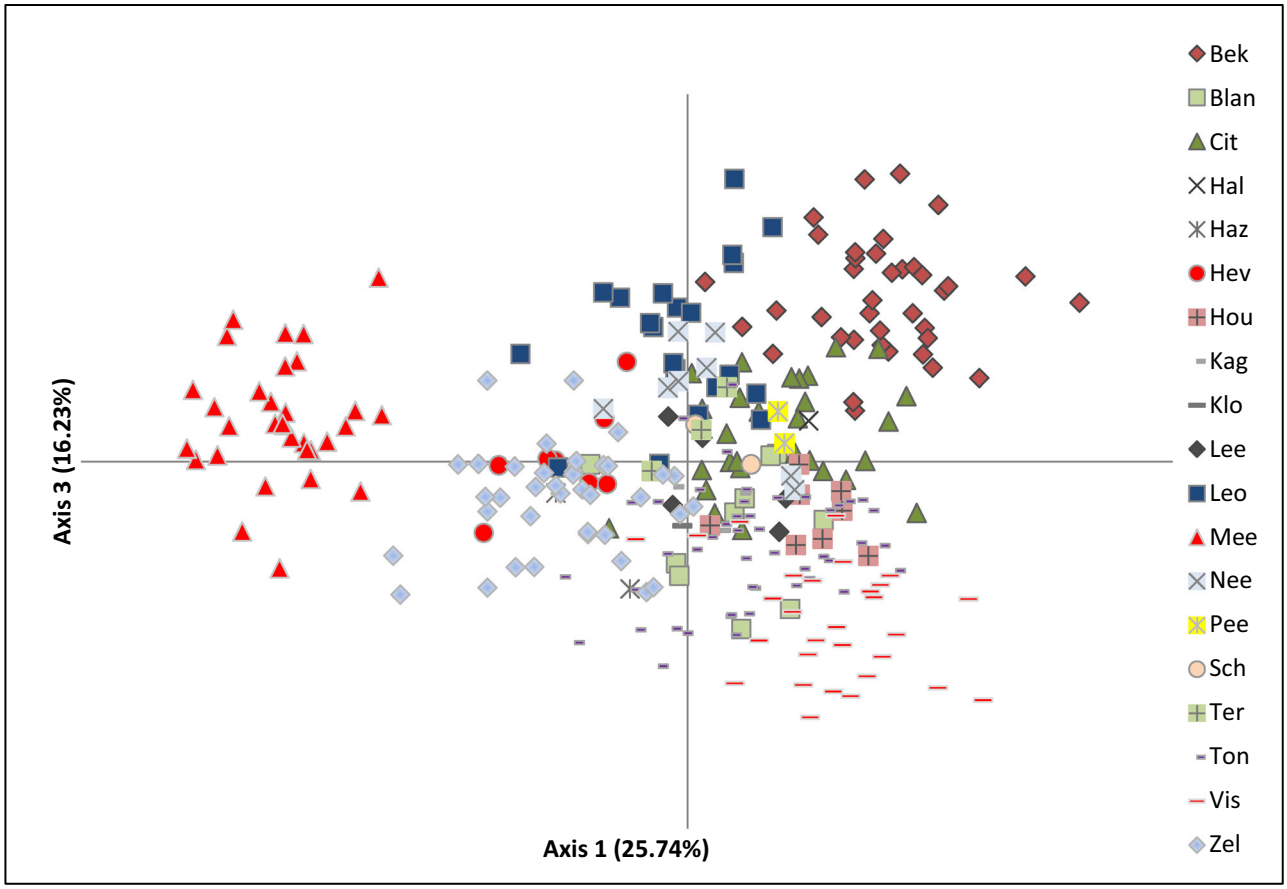
Ton
Vis
Zel


Axis 3 (16.23%)
Axis 1 (25.74%)
Bek
Blan
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Kag
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Leo
Mee
Nee
Pee
Sch
Ter
Ton
Vis
Zel

Table 3 Results of the linear regressions with estimated genetic diversity parameters [F_{IS}, H_j, PPL and log(G)] as response variables and fitness characteristics [log(area), seed germination speed and seed germination percentage] as exploratory variables based on 20 sampling populations of *Thymus pulegioides*

	Multiple R^2	F-statistic	DF	p value
F_{IS} ~ log(area)	0.1759	3.63	17	0.07382
H_j ~ log(area)	0.2722	6.36	17	0.02196*
PPL ~ log(area)	0.3238	9.62	17	0.006487*
Log(G) ~ log(area)	0.5111	17.77	17	0.0005813**
F_{IS} ~ seed germination speed	0.3462	6.88	13	0.02103*
H_j ~ seed germination speed	0.1011	1.46	13	0.2481
PPL ~ seed germination speed	0.1995	3.24	13	0.09514
Log(G) ~ seed germination speed	0.4827	12.13	13	0.004045**
F_{IS} ~ seed germination %	0.362	7.38	13	0.01765*
H_j ~ seed germination %	0.0939	1.35	13	0.2666
PPL ~ seed germination %	0.1886	3.022	13	0.1057
log(G) ~ seed germination %	0.462811	11.20	13	0.005258

F_{IS} estimated mean inbreeding coefficient, *H_j* expected heterozygosity, *PPL* percentage of polymorphic loci at the 5 % level, *G* the number of genets, *Area* area covered by the population, significant codes: * p values < 0.05, ** p value <0.005

microsatellites in calculating inbreeding and relatedness coefficients. However, the loss of information could be counterbalanced by a high number of polymorphic AFLP loci (Dasmahapatra et al. 2008) such as found in the present study.

Conservation of *T. pulegioides* is likely to depend on the simultaneously restoration of genetic diversity and habitat quality. Unfortunately, restoring historical dispersal processes and vectors to counteract genetic drift, such as movement of grazing sheep, is likely unfeasible in the current landscape. Genetic replenishment via the seed bank is also unlikely as *T. pulegioides*, like most grassland specialists, is generally absent in the seed bank (Bossuyt and Honnay 2008; Thompson et al. 1997). Maintaining large populations, free from the effects of genetic drift, may prove to be the only key to ensure long term persistence of characteristic grassland species. Therefore, following the restoration of suitable habitat, assisted dispersal of seed from neighboring local relic populations into existing populations and into restored suitable habitat may be required to counteract the effects of genetic drift and to successfully restore populations of target grassland species.

Data accessibility

The data set supporting the results of this article is available in the Dryad repository: Vanden Broeck An, Ceulemans Tobias, Kathagen Gunter, Hoffmann Maurice, Honnay Olivier and Mergeay Joachim. Dispersal constraints for the conservation of the grassland herb *Thymus pulegioides* L. in a highly fragmented agricultural landscape. Dryad. doi:10.5061/dryad.kb175.

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